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An abundant and novel protein of 22 kDa (SM22) is widely distributed in smooth muscles

Purification from bovine aorta

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Using a rabbit polyclonal-antibody preparation directed against the chicken gizzard protein, we demonstrated by immunoblotting the presence of the 22 kDa protein (SM22) in a variety of chicken smooth-muscle-containing organs, including uterus, intestine, gizzard, oesophagus and aorta. Protein SM22 was present in only trace amounts in brain, liver and heart, and could not be detected in chicken breast muscle. The antibody preparation did not cross-react with extracts of bovine aorta. However, the presence of SM22 as a major component in bovine aorta and pig carotid was demonstrated by its co-migration with the purified chicken gizzard protein on one- and two-dimensional polyacrylamide electrophoretic gels. Its molar abundance relative to actin was estimated to be 0.9:6.0 and 1.4:6.0 for bovine aorta and pig carotid respectively. Like the chicken gizzard protein, it separates on pH-gradient electrophoresis into at least three variants, α, β and γ, with similar apparent M_r. Purification of the aorta SM22 showed it to have a similar amino acid composition to the chicken gizzard protein. We conclude that SM22 is widely distributed and an abundant and unique protein component of smooth-muscle tissues of birds and mammals.

INTRODUCTION

The regulation of the contractile events in smooth muscle is not as clearly understood as that in skeletal and cardiac tissues. Although phosphorylation of the regulatory light chains in smooth muscle is known to be essential for actin activation of myosin Mg^{2+}-dependent ATPase (Walsh *et al.*, 1983; Asano & Stull, 1985) and to precede tension development, it does not necessarily correlate with tension maintenance (Driska *et al.*, 1981; Bárány *et al.*, 1985), indicating the possibility of additional control mechanisms. Although several alternative control mechanisms have been proposed (Ebashi, 1980; Chacko & Rosenfeld, 1982; Marston & Smith, 1985), it is clear that a fuller understanding of the properties of the proteins present in smooth muscles and the nature of their interactions is required. Towards this end we have (Lees-Miller *et al.*, 1987) isolated and characterized an abundant basic 22 kDa protein (SM22) present in chicken gizzards at a molar ratio to actin of 1:3. Although the cellular location and functional role of the protein are at present unknown, it is unique in its composition and properties, and by amino acid sequencing it was shown to resemble no other previously characterized protein (Pearlstone *et al.*, 1987). To assess further the importance of SM22 as a smooth-muscle component, we have in the present work demonstrated its presence in a variety of chicken smooth muscles by immunoblotting. Its presence in bovine aorta and pig carotid has been shown by two-dimensional and SDS/polyacrylamide-gel electrophoresis and its molar ratio to actin estimated to be 0.9:6.0 and 1.4:6.0 respectively. A procedure for purification of SM22 from bovine aorta is described, and its amino acid composition was shown to be similar to that of the chicken gizzard protein. We conclude that SM22 is widely distributed in smooth muscles as a major protein component.

MATERIALS AND METHODS

Preparation of antibodies and immunoblotting

SM22α, the α isoform of chicken gizzard SM22, was prepared as previously described (Lees-Miller *et al.*, 1987). For the raising of polyclonal antibodies, 200 μg of SM22α dissolved in 500 μl of water and 500 μl of Freund's complete adjuvant was injected into a rabbit, half subcutaneously and half intramuscularly. Further injections of 200 μg in Freund's incomplete adjuvant were at 10-day intervals for 3 months. Production of antibodies was monitored by enzyme-linked immunoabsorbent assay (Engvall, 1980), followed by their isolation on a Protein A affinity column. Immunoblotting was by the method of Towbin *et al.* (1979). SDS/urea extracts of chicken tissues, prepared as described by Lees-Miller *et al.* (1987), were electrophoresed on SDS/13%-polyacrylamide gels, in the buffer system of Laemmli (1970). After electrophoretic transfer to nitrocellulose (Schleicher & Schuell) and incubation with anti-SM22α antibody at a dilution of 1:5000, the reactive proteins were detected with goat anti-rabbit IgG–alkaline phosphatase (Sigma) and a solution containing 1 mg of Fast Red TR salt (Sigma)/ml, 1 mg of naphthol AS-BI phosphate (Sigma)/ml, 0.1 M-Tris, pH 9.2, and 5 mM-$MgCl_2$ (O'Connor & Ashman, 1982).

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Electrophoresis and gel scanning

Bovine aortas and pig carotids were kindly given by Gainers (Edmonton). SDS/urea extracts were run on SDS/13%-polyacrylamide gels, and the relative amounts of actin, SM22 and tropomyosin were determined by using a Joyce–Loebl Chromoscan 3 scanning densitometer as described previously (Lees-Miller *et al.*, 1987). Relative staining intensities with Coomassie Blue were based on standard curves for rabbit skeletal-muscle actin and chicken gizzard tropomyosin and SM22. Aorta proteins were identified on the gels according to their relative abundance and migration properties, as described for actin, myosin and tropomyosin (Cohen & Murphy, 1978), vimentin and desmin (Gabbiani *et al.*, 1981) and caldesmon (Clark *et al.*, 1986). Filamin was tentatively identified by co-migration with its gizzard counterpart, and albumin was purified from the 70%-satd.-$(NH_4)_2SO_4$ supernatant described below and identified from its amino acid composition. In all cases the protein identities were checked by two-dimensional gel electrophoresis and/or purification properties. Two-dimensional gels were run with non-equilibrium pH-gradient electrophoresis in the first dimension as described by O'Farrell *et al.* (1977).

Purification of SM22 from bovine aorta

External fat and connective tissue were removed from chilled aortas which were then minced in a grinder with 2.5 mm holes. The initial stages of purification were as described for chicken gizzards (Lees-Miller *et al.*, 1987) up to and including CM-cellulose chromatography at pH 4.7 (see Fig. 5). Since the aorta protein was not homogeneous at this stage, a further purification step was carried out on a 2.5 cm × 20 cm column of Affi-gel Blue (Bio-Rad) at pH 8.0 (see Fig. 6).

RESULTS

Detection of SM22 in SDS/urea extracts of various organs

We have used the immunoblotting procedure of Towbin *et al.* (1979) to investigate the presence or absence of SM22 in SDS/urea extracts of a variety of organs from the chicken and of bovine aorta. Rabbit anti-SM22 antibodies were found to bind to a 22 kDa protein in extracts of chicken organs that contain smooth muscle (Fig. 1). These included intestine, uterus, gizzard, oesophagus and aorta. With overloading (results not shown), trace amounts of SM22 were observed in heart, brain and liver, but none was detected in breast muscle. Anti-SM22 antibodies also cross-reacted with a 70 kDa protein in oesophagus which we have been unable to identify. The antibodies to gizzard SM22 did not cross-react with an SDS/urea extract of bovine aorta or with purified SM22 from this source (Fig. 2). A very weak cross-reaction with the heavily overloaded actin band was seen in the whole extracts in Fig. 2. This anomaly was not seen with the actin standard in Fig. 2 (lane 4) or in Fig. 1.

Presence of SM22 in mammalian arteries

SDS/urea extracts of bovine aorta and pig carotid arteries exhibited a major 22 kDa band (SM22) when electrophoresed on SDS/polyacrylamide gels (Fig. 3). The molar proportions of actin: SM22: tropomyosin are 6.0:0.9:1.0 in bovine aorta and 6.0:1.4:1.0 in pig carotid. This may be compared with proportions of 6.5:2.0:1.0 in chicken gizzard (Lees-Miller *et al.*, 1987). SM22 was also detected in non-equilibrium, two-dimensional gels of urea extracts from mammalian arteries (Fig. 4). When the most basic variant, α, of

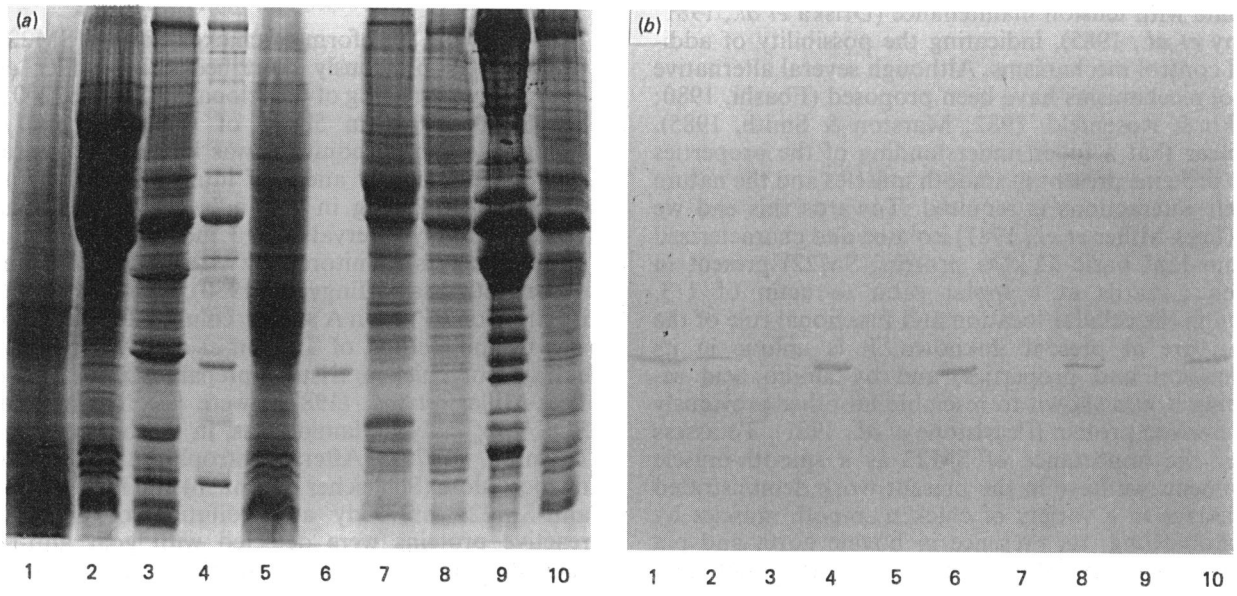

Fig. 1. Immunoblot of chicken organ extracts

(*a*) SDS/10–18%-polyacrylamide gel of chicken organ SDS/urea extracts, stained with Coomassie Blue: intestine (1), uterus (2), heart (3), gizzard (4), liver (5), SM22 (6), brain (7), oesophagus (8), skeletal muscle (9), aorta (10). (*b*) Nitrocellulose transfer of a gel with the same composition as in (*a*) and immunostained for SM22 as described in the Materials and methods section. Sample loadings are approx. 50 μg/lane, except for SM22 (0.5 μg), gizzard (15 μg) and skeletal muscle (100 μg).

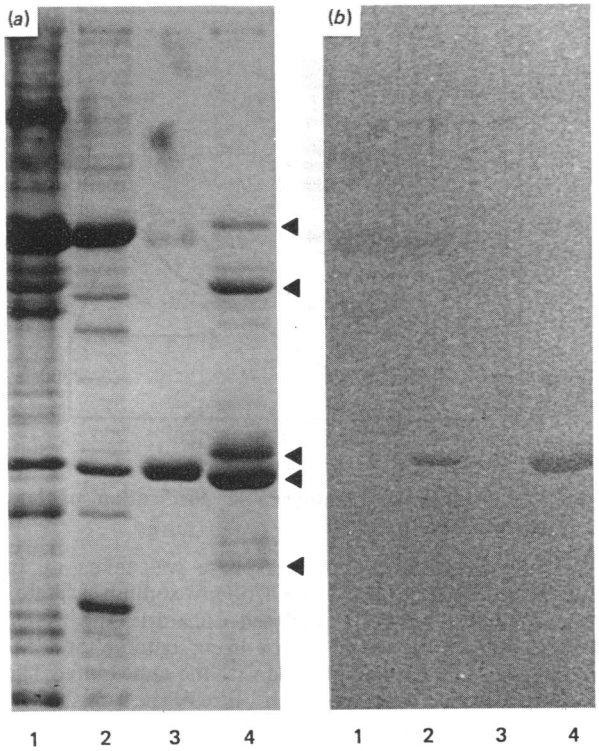

Fig. 2. Immunoblot of bovine aorta extract

Nitrocellulose transfers are shown of 13% -polyacrylamide gels of (1) bovine aorta (40 μg), (2) chicken gizzard (20 μg), (3) aorta SM22 (1 μg), and (4) standards (arrowheads), from top to bottom: actin (M_r 42000), rabbit skeletal-muscle troponin T (38000), troponin I (24000), gizzard SM22 (22000) and troponin C (17000). (*a*) Stained with Amido Black; (*b*) immunostained for SM22.

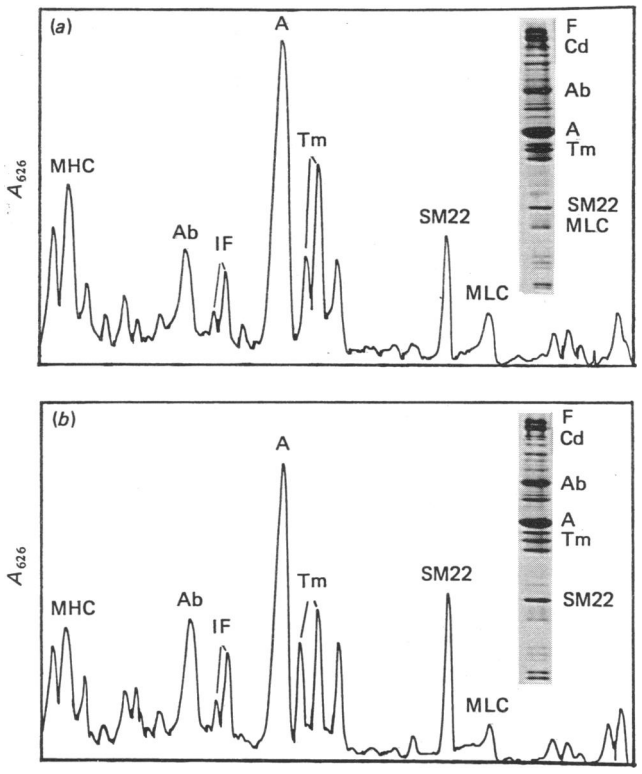

Fig. 3. Densitometric scans of vascular tissue extracts

SDS/urea extracts of bovine aorta (*a*) and porcine carotid (*b*) were run on SDS/13% -polyacrylamide gels. Filamin (F), myosin heavy chain (MHC), caldesmon (Cd), albumin (Ab), intermediate-filament proteins vimentin and desmin (IF), actin (A), tropomyosin (Tm) and myosin light chain (MLC) were identified as described in the Materials and methods section. The insets are photographs of the gels that were scanned.

gizzard SM22 was co-electrophoresed with a 9 M-urea extract of pig carotid it was indistinguishable, in both apparent M_r and pI, from the most basic variant of SM22 in the carotid tissue. The pig carotid SM22, like the gizzard SM22, appears to consist of at least three variants, designated α, β and γ in order of decreasing basicity. The relative constants of the β and γ components in this tissue is higher (proportions $\alpha:\beta:\gamma \sim 2.5:2:1$) than in the chicken gizzard (ratio of 14:5:1) (Lees-Miller *et al.*, 1987). For bovine aorta, the $\alpha:\beta:\gamma$ ratio was also $\sim 2.5:2:1$, and a still more acidic variant, δ (Fig. 4*a*, inset), was detected when higher loadings of the extract were applied to the two-dimensional gel. This variant was not seen in the chicken gizzard extracts.

To provide further evidence for the identity of SM22 in bovine aorta, we have purified a mixture of its α and β isoforms through an extension of the procedure used for its purification from chicken gizzards. This procedure was essentially the same as that previously described (Lees-Miller *et al.*, 1987) up to and including precipitation with 70% -satd. $(NH_4)_2SO_4$. At this stage the 22 kDa band was heavily contaminated with several other proteins, made up primarily of a 58 kDa component and a basic 20 kDa protein, which have not been identified (see Fig. 5, inset). Subsequent CM-cellulose chromatography (Fig. 5) achieves a considerable increase in purity of SM22, but, unlike the case with the gizzard extracts, leaves it still contaminated with the 58 and 20 kDa proteins. An additional purification step on Affi-gel Blue (Fig. 6), however, was successful in yielding an SM22 preparation that was > 90% pure, co-migrated with chicken gizzard SM22 on SDS/polyacrylamide gels (Fig. 2) and consisted primarily of a mixture of α and β isoforms (not shown). Amino acid analysis of this product (Table 1) indicated that its composition was very similar to that of the chicken gizzard SM22 α and β isoforms.

DISCUSSION

Many of the abundant smooth-muscle proteins have now been purified and characterized. Although most of this work was done initially with chicken gizzards, more recent purification and characterization of proteins such as myosin light-chain kinase (Hathaway *et al.*, 1985), caldesmon (Clark *et al.*, 1986), actin (Strzelecka-Golaszewska *et al.*, 1985) and myosin (Chacko & Rosenfeld, 1982) from mammalian vascular smooth muscle is indicative of the growing interest in this physiologically important tissue. Indeed, the first observation that myosin light-chain phosphorylation does not correlate with maintenance of tension (Driska *et al.*, 1981; Bárány *et al.*, 1985) in smooth muscle was made with strips of vascular tissue. These findings indicated the requirement for a second control mechanism in smooth muscle. Some possibilities that have been proposed, but

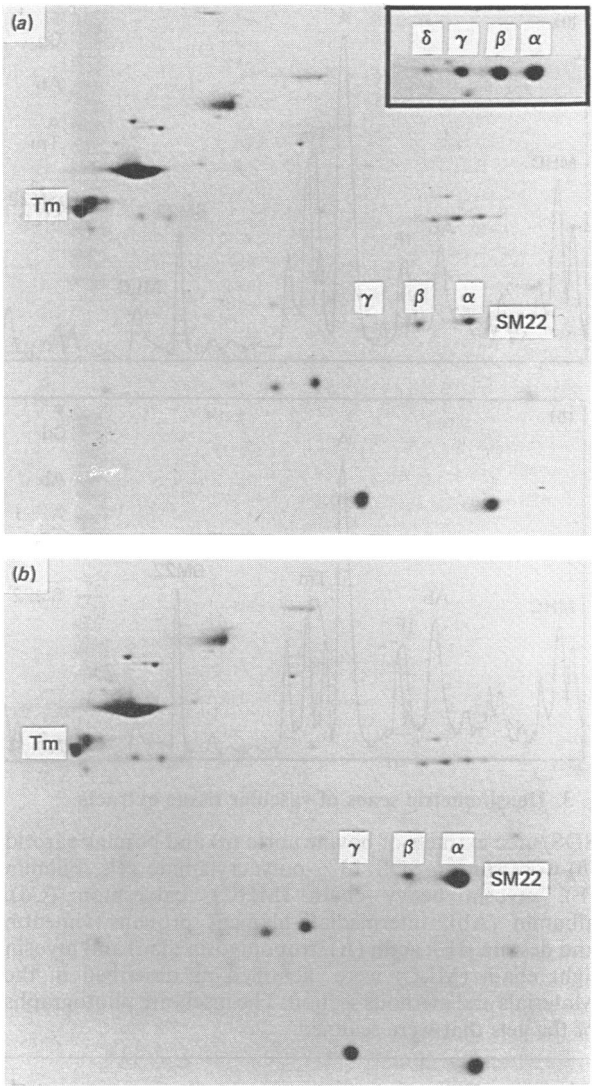

Fig. 4. Two-dimensional gels of a 9 M-urea extract of pig carotid (*a*) and pig carotid with added chicken gizzard SM22 *a* (*b*)

The horizontal dimension is non-equilibrium isoelectric focusing carried out for 3000 V·h in the presence of pH 3–10 Ampholines (LKB). The vertical dimension is SDS/14%-polyacrylamide gel electrophoresis. The inset is SM22 cut out from a two-dimensional gel of bovine aorta: α, β, γ and δ indicate the variants of SM22, from most basic to most acidic. Abbreviation: Tm, tropomyosin.

not proven, include binding of Ca^{2+} to myosin (Chacko & Rosenfeld, 1982), thin-filament-linked control by leiotonin (Ebashi, 1980) and a calmodulin-linked Ca^{2+} regulation by caldesmon (Marston & Smith, 1985).

Surprisingly, little attention has been paid to the possibility that low-M_r (below 30000) proteins other than the myosin light chains and calmodulin may play a role in the control of smooth-muscle contraction. However, Rapoport *et al.* (1982) demonstrated the phosphorylation of several low-M_r components in intact vascular smooth muscle, other than the myosin light chain. The possibility that these or other low-M_r protein components may contribute to the regulation of smooth-muscle metabolism deserves further investigation.

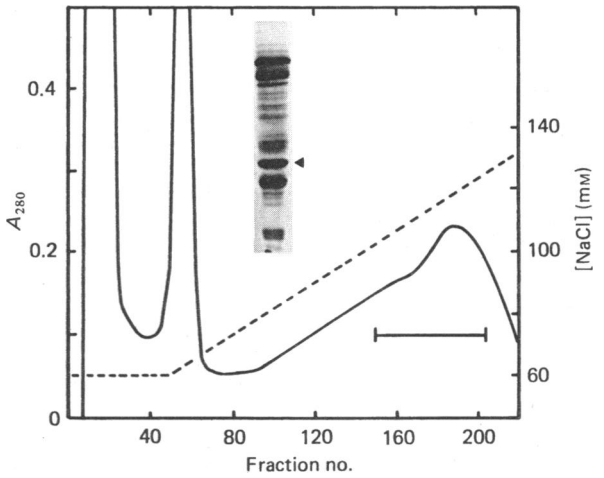

Fig. 5. CM-cellulose chromatographic purification of bovine aorta SM22

The 70%-satd.-$(NH_4)_2SO_4$ precipitate was taken up in 120 ml of buffer (60 mM-NaCl/50 mM-sodium acetate/1 mM-EGTA, pH 4.7), dialysed against the same buffer and applied to a 5 cm × 45 cm column of CM-cellulose, previously equilibrated with the same buffer. The column was then developed with 1 litre of the same buffer, followed by a 3 litre linear NaCl gradient (60–135 mM; ----). Flow rate, 120 ml/h; fraction size, 18 ml. Inset shows SDS/polyacrylamide gel of the 70%-satd.-$(NH_4)_2SO_4$ fraction applied to the column. Fractions were pooled as indicated.

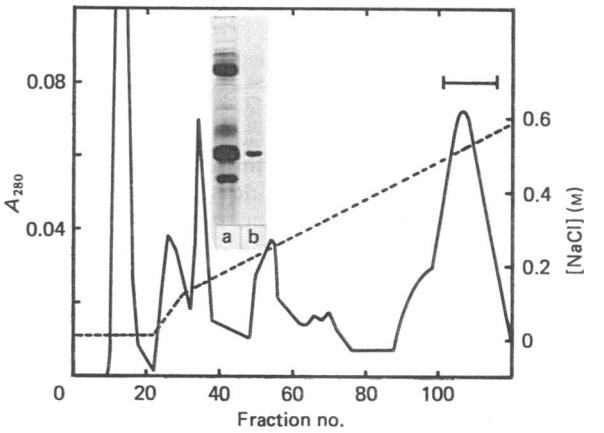

Fig. 6. Affi-gel Blue chromatographic purification of SM22

A sample (100 mg) of the dialysed and freeze-dried material pooled in Fig. 5 was dissolved in 30 ml of buffer [20 mM-Hepes (pH 8.0)/10 mM-NaCl/1 mM-EDTA/1 mM-dithiothreitol] and applied to a 2.5 cm × 20 cm column, previously equilibrated with the same buffer. The column was then developed with 250 ml of the same buffer followed by a 1 litre linear NaCl gradient (0.1–0.6 M; ----). Flow rate, 40 ml/h; fraction size, 10 ml. Inset gel lanes show material applied to the column (a) and that recovered from the pooled fractions (b).

Previously (Lees-Miller *et al.*, 1987) we have purified and characterized SM22 from chicken gizzard. This protein has an apparent M_r on SDS/polyacrylamide gels of 22000 and appears to be unlike any previously characterized protein. It is present in the molar

Table 1. Amino acid composition of SM22 from bovine aorta

The composition of aorta SM22, which was approximately a 3:2 mixture of α and β variants, was based on 11 alanine residues, in order to approach the sedimentation-equilibrium M_r of gizzard SM22α (22000). Samples of SM22 were hydrolysed for 24, 72 and 120 h. Values for serine and threonine were extrapolated to zero time. Valine and isoleucine were taken from the average of 72 and 120 h hydrolysates. Abbreviation: n.d., not determined.

	Composition (mol/mol)		
Residue	Aorta SM22	Gizzard SM22α*	Gizzard SM22β*
Asx	16	19	19
Thr	6.9	6.2	6.8
Ser	12	13	13
Glx	26	25	24
Pro	9.5	9.5	9.6
Gly	20	18	17
Ala	11	14	14
Val	16	14	14
Met	7.7	7.5	6.8
Ile	5.0	6.5	6.5
Leu	15	14	14
Tyr	5.7	6.0	5.8
Phe	6.0	7.1	6.9
His	3.2	3.2	3.3
Lys	16	18	17
Arg	11	9.2	8.4
Trp	n.d.	3.0	2.9
Half-Cys	n.d.	1.1	2.2

* See Lees-Miller *et al.* (1987).

proportions actin:SM22:tropomyosin of 6.5:2.0:1.0 and consists of three isoforms, in the molar proportions $\alpha:\beta:\gamma$ of 14:5:1. In the present study we have made polyclonal antibodies to gizzard SM22 and used them in the immunoblot procedure to test for the presence of SM22 in extracts from a variety of chicken organs. The results show that SM22 is present primarily in organs that contain abundant smooth-muscle tissue (Fig. 1) and are therefore consistent with SM22 being a smooth-muscle-specific protein. Immunohistochemical studies will, however, be required to substantiate this observation. We found that our rabbit anti-(chicken SM22) antibodies did not cross-react with extracts from bovine aorta (Fig. 2). One can, however, detect an abundant protein on two-dimensional gels of bovine aorta and pig carotid extracts with the same M_r and pI as SM22 from chicken gizzard (Fig. 4). We have purified SM22 from bovine aorta, using a procedure almost identical with that used for gizzard SM22 (Fig. 5). However, the presence of major contaminants after CM-cellulose chromatography in the aorta preparation (Fig. 5) made necessary a further purification step by Affi-Gel Blue column chromatography (Fig. 6).

Amino acid analysis of a mixture of α and β isoforms of aorta SM22 (Table 1) indicates that this protein is similar to gizzard SM22, despite its lack of antibody cross-reactivity. The isoform distribution of vascular and gizzard SM22 is also similar, although the more acidic β and γ subunits are relatively more abundant in vascular tissue ($\alpha:\beta:\gamma$, 2.5:2:1). A fourth, still more acidic, variant (δ), not detected in chicken gizzard, is present in aorta. For the chicken gizzard SM22 isoforms, no evidence was obtained that these were due to different phosphorylated forms (Lees-Miller *et al.*, 1987). However the possibility that this could account for the different isoforms of SM22 in the vascular tissues cannot be excluded at this time. The relative molar abundance of SM22 compared with actin and tropomyosin in vascular tissue is about half that found in gizzard.

We conclude that SM22 is present as a major component in the smooth muscles of both birds and mammals. Although the function of SM22 remains unknown, its abundance suggests an important role in the physiology of smooth-muscle tissue.

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